

Serratia marcescens Induces Apoptotic Cell Death in Host Immune Cells via a Lipopolysaccharide- and Flagella-dependent Mechanism^{*[S]}

Received for publication, July 12, 2012; Published, JBC Papers in Press, August 2, 2012; DOI 10.1074/jbc.M112.399667

Kenichi Ishii[‡], Tatsuo Adachi[‡], Katsutoshi Imamura[‡], Shinya Takano[§], Kimihito Usui[‡], Kazushi Suzuki[§], Hiroshi Hamamoto[‡], Takeshi Watanabe[§], and Kazuhisa Sekimizu^{*1}

From the [‡]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033 and the [§]Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Niigata 950-2181, Japan

Background: The pathogenic mechanism of *Serratia marcescens* is poorly understood.

Results: *S. marcescens* kills immune cells via an lipopolysaccharide- and flagella-dependent mechanism.

Conclusion: *S. marcescens* suppresses innate immunity by killing immune cells.

Significance: This is the first evidence to suggest that *S. marcescens* evades the immune system.

Injection of *Serratia marcescens* into the blood (hemolymph) of the silkworm, *Bombyx mori*, induced the activation of c-Jun NH₂-terminal kinase (JNK), followed by caspase activation and apoptosis of blood cells (hemocytes). This process impaired the innate immune response in which pathogen cell wall components, such as glucan, stimulate hemocytes, leading to the activation of insect cytokine paralytic peptide. *S. marcescens* induced apoptotic cell death of silkworm hemocytes and mouse peritoneal macrophages *in vitro*. We searched for *S. marcescens* transposon mutants with attenuated ability to induce apoptosis of silkworm hemocytes. Among the genes identified, disruption mutants of *wecA* (a gene involved in lipopolysaccharide O-antigen synthesis), and *flhD* and *fliR* (essential genes in flagella synthesis) showed reduced motility and impaired induction of mouse macrophage cell death. These findings suggest that *S. marcescens* induces apoptosis of host immune cells via lipopolysaccharide- and flagella-dependent motility, leading to the suppression of host innate immunity.

Living organisms are continuously in danger of infection by environmental pathogens, such as soil bacteria. Environmental pathogens cause infectious diseases that can be particularly dangerous to aged humans and patients with chronic disease. The Gram-negative bacterium *Serratia marcescens* is an environmental pathogen that infects a wide range of hosts, such as plants, invertebrates, and vertebrates (1). In compromised humans, *S. marcescens* causes respiratory infection, urinary tract infection, meningitis, and sepsis (2). Strains of *S. marcescens* that are resistant to various antibiotics, including β -lac-

tam, aminoglycoside, and fluoroquinolone, have recently emerged (3). Although overcoming *S. marcescens* infection has gained clinical importance over the last 40 years, the underlying mechanism of the pathogenesis of *S. marcescens* remains poorly understood.

To investigate the virulence mechanisms of pathogens, infection experiments must be performed with the appropriate model animals. Invertebrates possessing simple biologic systems have recently gained attention as model hosts for studies of infectious diseases (4, 5). We previously postulated the usefulness of a bacterial infection model using the silkworm *Bombyx mori* due to their low cost and ease of handling (e.g. injection into either the bloodstream or the gut is possible, and each organ can be dissected for biochemical and pharmacologic experiments), and the absence of ethical problems associated with the use of mammalian models (6–8). Silkworms are killed by infection with human pathogens such as *Staphylococcus aureus*, and are cured by antibiotics clinically effective for humans (6, 7). Among mutant strains of *S. aureus* in which genes with unknown functions were disrupted, we previously identified novel virulence genes, *cvfA*, *cvfB*, and *cvfC*, by screening using the silkworm infection model (9, 10). Furthermore, the silkworm model is applicable for assessing the virulence of microorganisms isolated from environmental sources (11, 12). Therefore, we consider silkworms to be a suitable model host for examining the pathogenesis of *S. marcescens*.

Insects such as silkworms lack antibody-producing organs, but possess innate immune systems to combat infectious agents. Invertebrate innate immune systems are similar to those of mammals (13). In silkworms, immune reactions are categorized as either humoral or cellular. Humoral immunity is represented by the production of antimicrobial peptides (AMPs)² (14, 15), and cellular immunity includes phagocytosis of microorganisms by blood cells (hemocytes) (16). Our recent studies have focused on an insect cytokine named paralytic peptide (PP) that regulates both humoral and cellular immunity

^{*} This work was supported by a Grant-in-Aid for Young Scientists (B) (21790063) and a Grant-in-Aid for Japan Society for the Promotion of Science Fellows (21-10519) from JSPS. This study was supported in part by a Grant-in-Aid for Scientific Research (B) (20390021), a Grant-in-Aid for Scientific Research on Priority Areas (21022015), and grants from Genome Pharmaceuticals Co. Ltd.

[S] This article contains supplemental Methods and Figs. S1–S7.

¹ To whom correspondence should be addressed: 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: 81-3-5841-4820; Fax: 81-3-5684-2973; E-mail: sekimizu@mof.f.u-tokyo.ac.jp.

² The abbreviations used are: AMP, antimicrobial peptide; IPS, insect physiological saline; PP, paralytic peptide; Z, benzyloxycarbonyl; fmk, fluoromethyl ketone.

in silkworms (17, 18). Nakahara *et al.* (19) originally reported that PP has biologic activity such as paralysis accompanied by muscle contraction, and induces morphologic changes in silkworm hemocytes. Injection of bacterial cell wall components in the silkworm blood (hemolymph) induces the conversion of PP from an inactive precursor to the active form (17). Moreover, treatment with a cytotoxic reagent inhibits the *in vitro* PP activation triggered by microbial factors, suggesting that live hemocytes are required in the process (17). The active form of PP induces both the expression of AMP genes in the fat body and the promotion of hemocyte phagocytosis of bacteria (18). Thus, similar to mammalian cytokines, insect cytokine PP seems to be involved in global regulation of multiple immune responses. The common features of the innate immune systems between silkworms and mammals make the silkworm a suitable model for investigating *S. marcescens* infection. Here we describe that *S. marcescens* suppresses innate immune reactions by killing immune cells of silkworms and mice.

EXPERIMENTAL PROCEDURES

Animals, Bacteria, and Reagents—Silkworm eggs (Hu-Yo × Tukuba-Ne) were purchased from Ehime Sanshu (Ehime, Japan). Silkworm larvae were reared on an antibiotic-free artificial diet at 27 °C. C57BL/6J mice were purchased from CLEA Japan. MyD88 knock-out mice were provided by Dr. Shizuo Akira (University of Osaka), Dr. Kaori Denda-Nagai, Dr. Nobuaki Higashi, and Dr. Tatsuo Irimura (University of Tokyo). *S. marcescens* 2170 strain and a methicillin-susceptible *S. aureus* strain (MSSA-1) were harvested from Brain Heart Infusion broth (BD Biosciences) after overnight culture at 30 °C. Glucan from bakers' yeast was purchased from Oriental Yeast Co., Ltd. The glucan was suspended in saline and sonicated before use. The active form of PP was chemically synthesized, as described previously (19). SP600125, ML3403, and wortmannin, pharmacologic inhibitors of JNK, p38, and PI3K, respectively, were purchased from Calbiochem, and dissolved in DMSO. Ac-DEVD-CHO and Z-VAD-fmk, caspase inhibitors, were purchased from Sigma and BIOMOL, respectively.

Infection of Silkworm Larvae—Fifth instar larvae of day 2 were injected with 50 μ l of bacterial cells suspended in saline. The supernatant of the bacterial culture was prepared by centrifugation at 6000 $\times g$ for 5 min followed by filtration through Millex-GV 0.22- μ m Durapore membrane filters (Millipore). Heat-killed bacteria were obtained by autoclaving the bacteria at 121 °C for 20 min. Larvae were injected with various bacterial samples and kept at 27 °C without feeding, and survival rates were determined. Survival curves plotted using the Kaplan-Meier method were tested for significance using the log rank test.

Measurement of Hemocyte Viability—Bacterial suspension (50 μ l) and pharmacologic JNK inhibitor (1 mM, 50 μ l) were injected into the hemolymph through the dorsal surface of the silkworm. Larval legs were cut with scissors and the hemolymph was collected in ice-cold tubes. Approximately 0.2 ml of hemolymph was obtained per larva (day 2 of 5th instar). Ten microliters of each hemolymph sample was mixed with an equal volume of 0.1% trypan blue and immediately observed

under a microscope. The numbers of trypan blue-negative and -positive cells were counted using a cytometer.

Detection of the Phosphorylated Form of JNK—Twenty larvae (day 2 of 5th instar) were injected with saline or live *S. marcescens* cells and incubated at 27 °C for 30 min. Approximately 2 ml of hemolymph was collected in 8 ml of 1 mM benzamidine in phosphate-buffered saline (PBS). After centrifugation at 1000 $\times g$ for 5 min at 4 °C, the precipitated cells were lysed with sodium dodecyl sulfate sample buffer. Samples were subjected to SDS-PAGE, and the separated proteins were transferred onto Immobilon-P polyvinylidene fluoride membranes (Millipore). The membranes were sequentially soaked in 5% phosphoBLOCKER™ (Cell Biolabs) dissolved in Tris-buffered saline-Tween 20 (TBST; 20 mM Tris, 130 mM NaCl, 0.1% Tween 20, pH 7.6), anti-active JNK pAb (pTppY) (Promega number V793A) 1/1000 diluted in the blocking solution, and horseradish peroxidase-linked anti-rabbit antibody (GE Healthcare; 1/5000 diluted in the blocking solution). Bands were detected using Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life sciences) and Amersham Biosciences Hyperfilm ECL (GE Healthcare).

Detection of Caspase Activation in Hemocytes—Activation of caspase in hemocytes was detected with the NucView™ 488 Caspase-3 Assay Kit for Live Cells (Biotium number 30029) according to the manufacturer's instructions with slight modifications. Fifteen larvae (day 2 of 5th instar) were injected with 50 μ l of insect physiological saline (IPS; comprising 150 mM NaCl, 5 mM KCl, and 1 mM CaCl₂) or an overnight culture of *S. marcescens* suspended in IPS, and after 1.5 h the hemolymph was collected in ice-cold tubes containing 5 ml of collection buffer (1 mM benzamidine dissolved in IPS). The hemolymph was centrifuged at 400 $\times g$ for 5 min, and precipitated cells were suspended in IPS containing 10 μ M of the caspase inhibitor Ac-DEVD-CHO. The cells were transferred to a poly-L-lysine chamber slide and incubated at 27 °C for 15 min. Aliquots were removed and 400 μ l of NucView 488 caspase-3 substrate diluted in IPS was added. After incubation at 27 °C for 30 min, the cells were fixed in PBS containing 10% formaldehyde for 10 min. Aliquots were then removed and samples were mounted with Prolong® Gold antifade reagent with DAPI (Invitrogen number P36935). Cells were observed under a fluorescence microscope (Leica DFC300 FX). Cells with NucView 488-stained nuclei were detected.

Detection of Apoptotic Hemocytes—Apoptotic hemocytes were detected with a GFP-Certified™ Apoptosis/Necrosis Detection Kit (Enzo Life Sciences number ENZ-51002-25) according to the manufacturer's instructions with slight modifications. Ten larvae (day 2 of 5th instar) were injected with 50 μ l of IPS or an overnight culture of *S. marcescens* suspended in IPS, and after 1.5 h the hemolymph was collected in ice-cold tubes containing 5 ml of 1 mM benzamidine dissolved in IPS. The hemolymph was centrifuged at 400 $\times g$ for 5 min and washed again with the collection buffer. Precipitated hemocytes were suspended in 500 μ l of detection solution (1 \times binding buffer, apoptosis detection reagent containing Annexin V-EnzoGold (enhanced Cyanine 3) conjugate, necrosis detection reagent containing 7-AAD, and 10 μ M of a caspase inhibitor Ac-DEVD-CHO in IPS) and transferred to a poly-L-lysine-

S. marcescens Suppresses Innate Immunity

coated chamber slide (Iwaki number 4722-040). After 15 min at room temperature, aliquots were removed, and cells were fixed with 2% formaldehyde in PBS for 10 min. The cells were mounted and stained with DAPI as described above.

Muscle Contraction Assay—Measurement of muscle contraction activity in silkworm was described previously (17, 20). The intensity of the muscle contraction was expressed as the contraction value (C), calculated by measuring the maximum length of each specimen before (x cm) and after (y cm) the injection using the formula $(x - y)/x$ (17). To study the effects of preinjection of bacterial cells on glucan- and PP-dependent muscle contraction, 50 μ l of bacterial suspension was injected into the hemocoel of the larval specimen, and after 2 h, 100 μ l of glucan (50 μ g/ml) or 50 μ l of active PP (4 μ g/ml) was injected.

Measurement of Viability of Mouse Peritoneal Macrophages—Mice (C57BL/6J) were intraperitoneally injected with 100 μ l of 2% Brewer thioglycollate medium (Kanto Chemical Co., Inc.), and peritoneal macrophages were collected 3 days later. Macrophages were suspended in PBS (approximately 3×10^6 cells/ml) and incubated in tissue culture-treated 96-well plates for 0.5 to 1 h at 37 °C, and then the supernatant was discarded to remove unattached cells. A bacterial suspension (approximately 2×10^{10} cells/ml) was added and incubated with macrophages for 2 h at 37 °C. The numbers of trypan blue-negative and -positive cells were counted using a cytometer.

Construction of *S. marcescens* Transposon-inserted and Gene-disrupted Mutants—Transposon-inserted mutants were constructed by conjugation of parent *S. marcescens* 2170 and *Escherichia coli* SM10 λ pir harboring pUTmini-Tn5-Km1 plasmid as previously described (21, 22). Gene-disrupted mutants were constructed by homologous recombination using *pir*-dependent plasmid pFS100 or pFS200 (21, 23). See supplemental Methods for the PCR primers used in the construction of gene-disrupted mutants.

Analysis of LPS and Flagella Fractions of *S. marcescens*—LPS fraction of *S. marcescens* was prepared by ethanol precipitation as previously described (24). The LPS fraction was analyzed on a 15% SDS-polyacrylamide gel, and the gel was stained with Sil-best stain One (Nacalai Tesque). The flagella fraction of *S. marcescens* was prepared as previously described (25). The flagellin protein was separated on a 12.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue.

Motility Assay of *S. marcescens* Mutants—Culture medium (10 g of Bacto-Trypton (BD Biosciences) and 5 g of NaCl (Wako Pure Chemical Industries, Ltd.) dissolved in 1 liter of reverse osmosis water) containing 0.4% agar (Nacalai Tesque) was autoclaved for 20 min at 121 °C. Agar medium was poured into a sterile Petri dish and dried for 20 min at room temperature without the lid. Overnight culture (1 μ l) of each bacterial strain was spotted on the soft agar and dried for 10 min. The dish was then covered and incubated for 9 h at 30 °C.

Determination of LD₅₀ of *S. marcescens* against Silkworm Larvae—Fifth instar larvae of day 2 were injected with 2-fold serially diluted suspension of *S. marcescens* and incubated at 27 °C. After 16 h, the number of viable larvae was counted. LD₅₀ (the lethal dose for 50% of the larvae) values were determined using the Reed-Muench method.

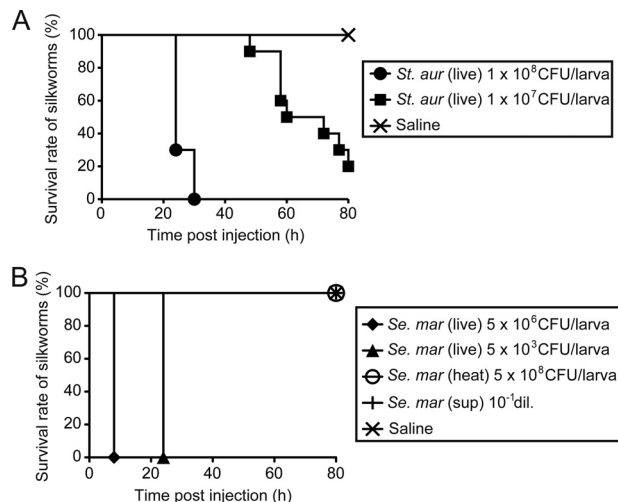


FIGURE 1. Killing of silkworms by *S. aureus* or *S. marcescens*. A, overnight culture of *S. aureus* (1×10^8 or 1×10^7 cfu/larva) was injected into the hemolymph of 10 larvae, and the survival rates were determined. B, overnight culture of *S. marcescens* (5×10^6 or 5×10^3 cfu/larva), autoclaved *S. marcescens* cells (5×10^8 cfu/larva), or a 10-fold dilution of the culture supernatant of *S. marcescens* was injected into the hemolymph of 10 larvae, and the survival rates were determined. The experiment was repeated three times with similar results.

RESULTS

Killing of Silkworms by *S. marcescens*—Various organisms pathogenic to humans killed silkworms when injected into the hemolymph (6, 12). Among the pathogenic bacteria previously tested in the silkworm infection model, *S. marcescens* had exceptionally high virulence in silkworms. Injection of 1×10^8 cells/larva of *S. aureus* was required to kill silkworms within 36 h (Fig. 1A). In contrast, 5×10^6 or 5×10^3 *S. marcescens* cells/larva killed silkworms within 12 or 36 h, respectively (Fig. 1B). Neither a 1/10-fold dilution of the filtrated culture supernatant nor heat-killed *S. marcescens* cells killed silkworms within 80 h (Fig. 1), indicating that the high virulence of *S. marcescens* in silkworms requires live bacterial cells.

Killing of Hemocytes in the Silkworm Hemolymph by *S. marcescens*—In general, the ability to attack host immunity is important for pathogens to exert full virulence. We hypothesized that *S. marcescens* cells effectively kills silkworms by impairing the host immune system. Insect immune responses are divided into two categories: humoral immunity, such as AMP production, and cellular immunity, including phagocytosis by hemocytes. The resistance of *S. marcescens* to insect AMPs is comparable with that of other pathogens (26). Therefore, we considered the possibility that *S. marcescens* evades cellular immune responses.

First, we tested the cytotoxicity of *S. marcescens* in hemocytes from silkworm hemolymph. Three hours after injecting the *S. marcescens* suspension, most of the isolated hemocytes were stained with trypan blue, an indicator of cell death (Fig. 2A). In contrast, injection of either the filtrated culture supernatant of *S. marcescens*, heat-killed *S. marcescens*, or a suspension of live *S. aureus* did not increase the ratio of trypan blue-positive cells in silkworm hemocytes (Fig. 2A). These results suggest that infection of silkworms with live *S. marcescens* kills hemocytes.

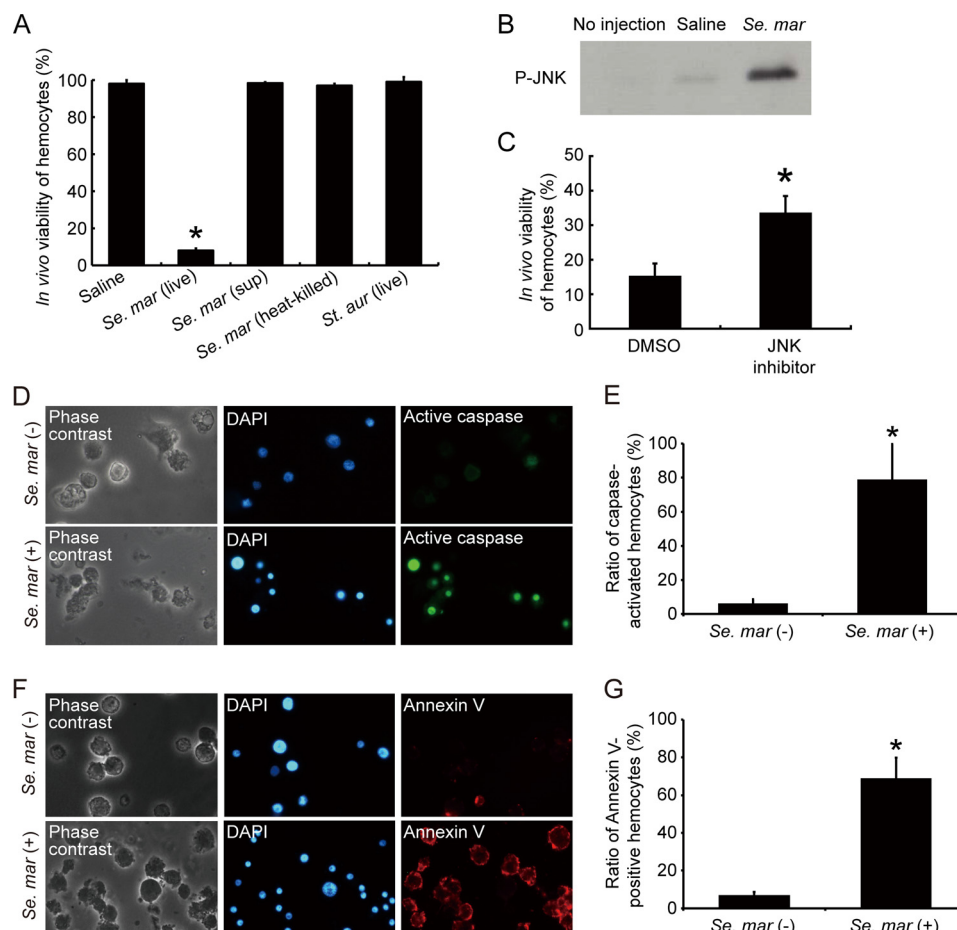


FIGURE 2. Induction of hemocyte death in vivo by *S. marcescens*. *A*, killing of silkworm hemocytes *in vivo* by *S. marcescens*. Saline, a suspension of live *S. marcescens* (1×10^{10} cfu/ml), a culture supernatant of *S. marcescens*, a suspension of autoclaved *S. marcescens*, or a suspension of live *S. aureus* (2×10^{10} cfu/ml) was injected into the hemolymph of 3 larvae (50 μ l/larva), and after 3 h the hemocytes were isolated and stained with trypan blue. The ratio of trypan blue-negative hemocytes was determined. Statistical significance was determined by Student's *t* test (*, $p < 0.05$). *B*, *in vivo* activation of JNK in silkworm hemocytes by *S. marcescens*. Saline or *S. marcescens* suspension (1×10^{10} cfu/ml) was injected into the hemolymph of 20 larvae (50 μ l/larva), and the hemocytes were collected after 30 min. The phosphorylated form of JNK in the samples, extracted from equal numbers of hemocytes (6×10^6 cells/50 μ l), was detected by Western blot analysis. The experiment was repeated three times and representative data are shown. *C*, effect of JNK inhibitor on *in vivo* hemocyte killing by *S. marcescens*. Fifty microliters of 10% DMSO (a solvent) or the JNK inhibitor SP600125 (1 mM) was preinjected into 3 larvae (50 μ l/larva), and 30 min later either saline or *S. marcescens* suspension (2×10^{10} cfu/ml) was injected (50 μ l/larva). After 3 h, the hemocytes were collected and cell viability was determined by trypan blue staining. Data represent mean \pm S.D. of 3 larvae. Statistical significance was determined by Student's *t* test (*, $p < 0.05$). *D* and *E*, NucView 488 staining of caspase-activated hemocytes in *S. marcescens*-infected silkworms. IPS or *S. marcescens* suspension (1×10^{10} cfu/ml) was injected into the hemolymph of 15 larvae (50 μ l/larva), and the hemocytes were collected after 1.5 h. Caspase-activated cells were detected by using NucView 488-caspase substrate conjugate. When the substrates are cleaved by active caspases, they release DNA-binding dyes that stain the cell nucleus, which produces a staining pattern similar to DAPI. *F* and *G*, Annexin V staining of apoptotic hemocytes in *S. marcescens*-infected silkworms. IPS or *S. marcescens* suspension (1×10^{10} cfu/ml) was injected into the hemolymph of 10 larvae (50 μ l/larva), and the hemocytes were collected after 1.5 h. Cell nuclei were stained with DAPI. Cells undergoing apoptosis were detected using Annexin V-EnzoGold (enhanced Cyanine 3) conjugate, which binds to phosphatidylserine on the outer plasma membrane. Five to seven microscopic areas were observed and 300 to 400 cells were counted to measure the ratio of NucView 488- (*E*) or Annexin V- (*G*) positive cells. The statistical significance of differences was determined using Student's *t* test (*, $p < 0.05$).

The apoptosis signaling pathways are well conserved among species (27, 28). Factors such as c-Jun NH₂-terminal kinase (JNK) and caspases are involved in apoptotic processes in *Drosophila* and mammals (29, 30). Therefore, we examined whether *S. marcescens* induces cell death in silkworm hemocytes via activation of these apoptotic factors. We obtained hemocytes from silkworms injected with either saline or *S. marcescens*, and prepared cell homogenates. Western blot analysis revealed that injection of *S. marcescens* induced the phosphorylation of JNK within 30 min (Fig. 2*B*). We then tested the effect of SP600125, a pharmacologic JNK inhibitor, on hemocyte viability after infection by *S. marcescens*. Preinjection of SP600125 attenuated hemocyte killing, as assessed by trypan blue staining (Fig. 2*C*). Furthermore, we examined whether

caspase was activated and apoptosis occurred in hemocytes of *S. marcescens*-injected silkworms. Staining with NucViewTM 488-caspase substrate, a conjugate of a caspase substrate DEVD peptide and a DNA-binding dye that stains the nucleus when cleaved by active caspases, revealed that caspase-activated hemocytes were increased by injection of *S. marcescens* (Fig. 2, *D* and *E*). In addition, the surfaces of most hemocytes collected from *S. marcescens*-injected silkworms were stained with the Annexin V-Cyanine 3 fluorophore conjugate (Fig. 2, *F* and *G*). Annexin V is widely used to detect apoptosis because it has a high affinity to phosphatidylserine, which is translocated from the inner to the outer plasma membrane of apoptotic cells. *S. marcescens*-infected cells were also stained with 7-AAD, a dye that binds to DNA in membrane-permeabilized cells, suggest-

S. marcescens Suppresses Innate Immunity

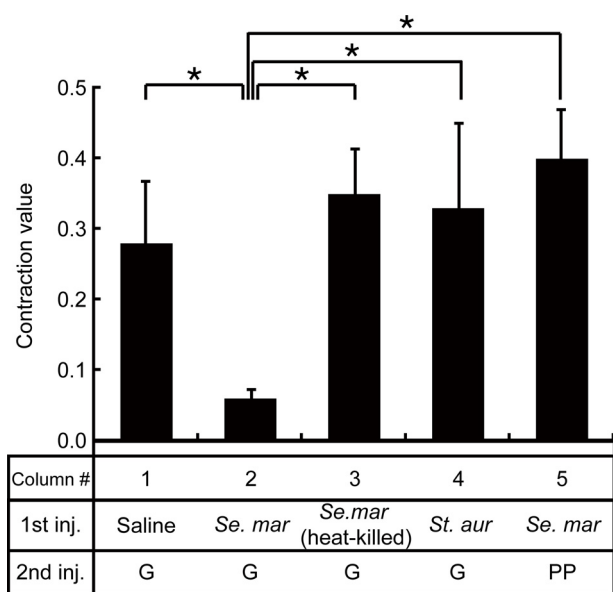


FIGURE 3. Inhibitory effect of *S. marcescens* on glucan-induced contraction of silkworm larval muscle specimen. Suspensions of live *S. marcescens* (2×10^{10} CFU/ml), autoclaved *S. marcescens*, or live *S. aureus* (1×10^{10} cfu/ml) were preinjected into larval muscle specimens (50 μ l/larva). After 2 h, 100 μ l of glucan (50 μ g/ml) (G) or 50 μ l of active paralytic peptide (4 μ g/ml) (PP) was injected, and the contraction value was measured. Data represent mean \pm S.D. of 3 or 4 specimens. Statistical significance was determined by Student's *t* test (*, $p < 0.05$).

ing that most of the cells were in the late phase of apoptosis (data not shown). Taken together, these findings suggest that *S. marcescens* infection induces the activation of apoptotic factors, followed by hemocyte death in silkworms.

Suppression of Cytokine Activation via Hemocyte Killing by *S. marcescens*—We previously reported that PP regulates multiple immune responses and contributes to host defense in silkworms (17, 18). Activation of PP is stimulated by pathogen cell wall components, such as glucan, injected in the hemolymph (17). In this process, hemocytes seem to be required for the recognition of pathogenic components and the subsequent production of reactive oxygen species resulting in PP activation (17). Active PP has a paralytic effect on silkworm larvae accompanied by muscle contraction (17). Thus, injection of glucan into the hemolymph of larval muscle specimens induces contraction via PP activation (17). To examine whether *S. marcescens* inhibits PP activation under conditions in which hemocytes in a larval specimen are killed by *S. marcescens*, we first injected a suspension of live *S. marcescens* cells, and then glucan, a representative PP-activation stimulant, and measured the muscle contraction strength in silkworm larval specimens. When the specimens were injected with saline and then with glucan (Fig. 3, column 1), they exhibited strong muscle contraction (contraction value (C) = 0.28, see “Experimental Procedures” for details regarding the C-value calculation), which seemed to result from PP activation in the hemolymph. Preinjection of live *S. marcescens* cells suppressed glucan-induced muscle contraction to C = 0.06 (Fig. 3, column 2), whereas heat-killed *S. marcescens* cells (Fig. 3, column 3) and live *S. aureus* cells (Fig. 3, column 4) did not. Thus, the effects of live *S. marcescens* cells, heat-killed *S. marcescens* cells, and live *S. aureus* cells on the suppression of glucan-induced muscle con-

traction (Fig. 3), an indicator of PP activation, were very consistent with their *in vivo* toxicity to hemocytes (Fig. 2A). On the other hand, when specimens pretreated with live *S. marcescens* were further injected with the purified active form of PP, the C values were still high (Fig. 3, column 5). Therefore, the responsiveness of muscle specimens to active PP seemed to be retained even under conditions in which hemocytes are killed by live *S. marcescens*. These results suggest that inhibition of glucan-dependent muscle contraction in silkworm specimens by live *S. marcescens* was due to the death of the hemocytes upstream of PP activation.

Induction of Apoptosis of Host Immune Cells *In Vitro* by *S. marcescens*—As described above, hemocytes in silkworms injected with *S. marcescens* were killed via JNK activation. We thus hypothesized that *S. marcescens* acts directly on hemocytes to induce apoptosis. To test this hypothesis, we analyzed the effect of *S. marcescens* on the viability of hemocytes isolated from the hemolymph of silkworms *in vitro*. When hemocytes were incubated with live *S. marcescens*, hemocyte viability decreased after 1.5 h (Fig. 4, A and B). Consistent with the *in vivo* experiments (Fig. 2A), heat-killed *S. marcescens* and live *S. aureus* did not affect hemocyte viability *in vitro* within 3 h (Fig. 4B). We then tested the effects of a JNK inhibitor on the cytotoxic effect of *S. marcescens* on hemocytes. Hemocytes isolated from silkworms were pretreated *in vitro* with SP600125 and incubated with *S. marcescens* for 3 h. Based on trypan blue staining, hemocytes treated with SP600125 were more viable than control hemocytes (Fig. 4C). Moreover, pretreatment of hemocytes with pharmacologic caspase inhibitors (Ac-DEVD-CHO and Z-VAD-fmk) suppressed hemocyte death induced by *S. marcescens* *in vitro* (Fig. 4D). In contrast, inhibitors of other cell-signaling factors such as p38 MAPK and PI3K were not effective against *S. marcescens*-induced hemocyte killing (supplemental Fig. S1). These findings suggest that *S. marcescens* acts directly on silkworm hemocytes and activates apoptotic factors such as JNK and caspase, which leads to hemocyte death.

We then tested whether *S. marcescens* induced cell death in mammalian immune cells. Similar to silkworm hemocytes, mouse peritoneal macrophages incubated with live *S. marcescens* were killed (Fig. 4E). Moreover, treatment with a JNK inhibitor (SP600125) or caspase inhibitors (Ac-DEVD-CHO or Z-VAD-fmk) prior to incubation with *S. marcescens* increased the viability of mouse macrophages (Fig. 4E). These results suggest that *S. marcescens* induces JNK- and caspase-dependent apoptosis in mouse macrophages as well as in silkworm hemocytes.

Transposon Mutagenesis Screening of *S. marcescens* Genes Involved in Hemocyte Apoptosis—To identify the virulence genes required to induce apoptosis in the host immune cells, we then screened *S. marcescens* transposon mutants. Among a total of 1049 transposon mutant strains, we identified 16 strains with attenuated *in vitro* cytotoxicity to silkworm hemocytes (supplemental Fig. S2). The killing effects on silkworms, based on by the LD₅₀ were attenuated in these mutants; 4 strains (STM91, 447, 673, and 898) had a 30–110-fold increase in the LD₅₀, and the remaining 12 strains had a 2–4-fold increase in the LD₅₀ (data not shown). We determined the genome sequences near the insertion positions of transposons in these 16 strains. Fifteen ORFs in which the transposons were inserted either within or upstream are shown in Table 1 (see also

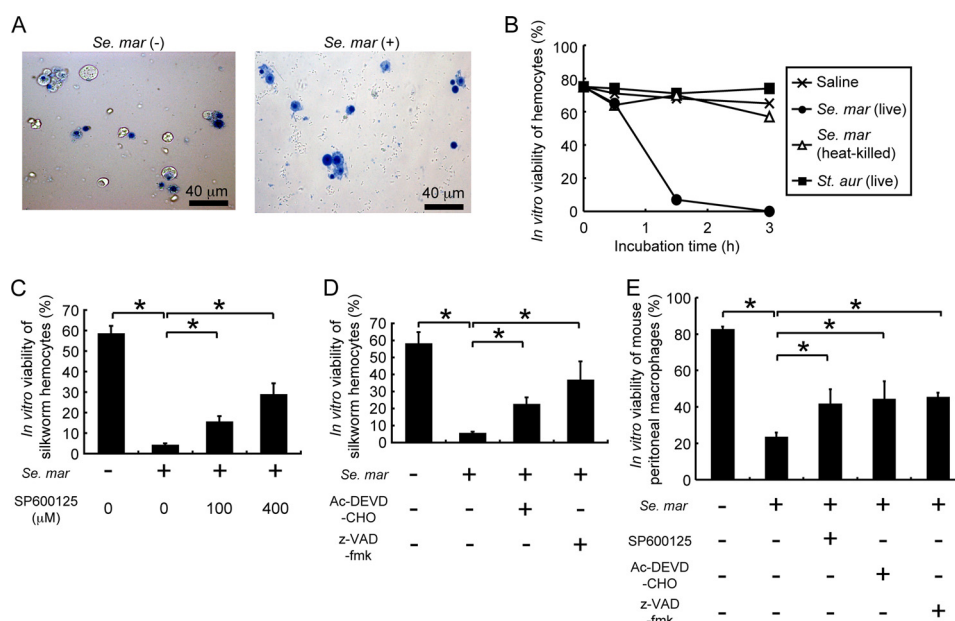


FIGURE 4. Induction of apoptosis of immune cells *in vitro* by *S. marcescens*. A, killing of silkworm hemocytes *in vitro* by *S. marcescens*. Hemocytes isolated from silkworm larvae were incubated with *S. marcescens* cells in saline, and after 3 h the cells were stained with trypan blue and observed under a microscope. B, time course of hemocyte killing by *S. marcescens*. Hemocytes (1×10^7 cells/ml) were incubated in saline with live cells of *S. marcescens* (1×10^9 cfu/ml), autoclaved cells of *S. marcescens*, or live cells of *S. aureus* (1×10^9 cfu/ml), and stained with trypan blue at the indicated time points. C, effect of a JNK inhibitor on hemocyte killing *in vitro*. Silkworm hemocytes suspended in PBS ($3-4 \times 10^6$ cells/ml) were supplied with 100 or 400 μ M SP600125, a pharmacologic JNK inhibitor. After 1 h, *S. marcescens* cells ($1-2 \times 10^8$ cfu/ml) were added to the hemocytes and the cells were incubated for 3 h. Hemocyte viability was determined by trypan blue staining. Data represent mean \pm S.D. of three experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.05$). D, effect of caspase inhibitors on hemocyte killing *in vitro*. Hemocytes suspended in PBS ($6-7 \times 10^6$ cells/ml) were supplied with 100 or 400 μ M Ac-DEVD-CHO, a pharmacologic JNK inhibitor, or 400 μ M Z-VAD-fmk, a pharmacologic inhibitor of caspase. After 1 h, *S. marcescens* cells ($1-2 \times 10^8$ cfu/ml) were added to the hemocytes and the cells were incubated for 3 h. Hemocyte viability was determined by trypan blue staining. Data represent mean \pm S.D. of three experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.05$). E, effects of inhibitors of apoptotic signaling factors (JNK and caspase) on killing of mouse macrophages *in vitro* by *S. marcescens*. Mouse peritoneal macrophages suspended in PBS ($3-4 \times 10^6$ cells/ml) were supplied with SP600125 (100 μ M), Ac-DEVD-CHO (200 μ M), or Z-VAD-fmk (400 μ M). After 1 h, *S. marcescens* cells ($1-2 \times 10^{10}$ cfu/ml) were added to the macrophages and the cells were incubated for 2 h. Macrophage viability was determined by trypan blue staining. Data represent mean \pm S.D. of three experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.05$).

TABLE 1
Genome analysis of *S. marcescens* transposon-inserted mutants with attenuated virulence against silkworm hemocytes

Strain no.	Gene with inserted transposon	Species with homologous gene	Identity %	Gene product function
STM91	<i>wecA</i>	<i>Serratia proteamaculans</i>	93	LPS O-antigen synthesis
STM162	PST family polysaccharide transporter	<i>Serratia odorifera</i>	86	Amino sugar metabolism
STM261	<i>citC</i>	<i>Escherichia coli</i>	94	Citrate metabolism
STM314	Phage integrase	γ - <i>Proteobacterium</i>	89	Lysogeny regulation
STM315	Hypothetical protein PROSTU_00814	<i>Providencia stuartii</i>	61	Unknown
STM316	AraC/XylS family transcriptional activator	<i>Xenorhabdus bovienii</i>	29	Resistance to antibiotics and heavy metals
STM394	Permease	<i>S. odorifera</i>	97	Membrane transport
STM396	Hypothetical protein ESCAB7627_2151	<i>Escherichia albertii</i>	49	Unknown
STM417	Hypothetical protein Sden_2787	<i>Shewanella denitrificans</i>	57	Unknown
STM447	<i>fliR</i>	<i>S. odorifera</i>	91	Flagellar formation (flagellar biosynthetic protein)
STM639	Hypothetical protein H16_A1750	<i>Ralstonia eutropha</i>	77	Unknown
STM673	<i>flgH</i>	<i>S. odorifera</i>	95	Flagellar formation (flagellar L-ring protein)
STM854	<i>bsmB</i>	<i>Serratia liquefaciens</i>	95	Biofilm formation
STM855	Protein kinase HdNI_03960	γ - <i>Proteobacterium</i>	89	Unknown
STM898	<i>flhD</i>	<i>S. marcescens</i>	100	Flagellar formation (flagellar transcriptional activator)

supplemental Fig. S3). Because the genomic sequence was not obtained for the remaining strain, STM149, further analysis of this strain was suspended. Among the determined ORFs, we focused on *wecA*, which is required for O-glycosylation of LPS, and *flhD* or *fliR*, which are essential in flagella biosynthesis. We constructed gene-disrupted *S. marcescens* mutants of *wecA*, *flhD*, or *fliR* (Δ *wecA*, Δ *flhD*, or Δ *fliR*, respectively) and analyzed their LPS and flagella structures. As expected, the LPS O-anti-

gen was abolished in Δ *wecA*, whereas that in Δ *flhD* and Δ *fliR* seemed to be intact (supplemental Fig. S4A). In addition, the *wecA* mutant lacked the 39-kDa flagellin protein (supplemental Fig. S4B), consistent with a previous report showing that LPS O-antigen ligase is required for flagella biosynthesis in the Gram-negative bacterium *Proteus mirabilis* (31). We next confirmed that Δ *wecA*, Δ *flhD*, and Δ *fliR* had impaired hemocyte killing ability compared with the parent strain (Fig. 5, A and B).

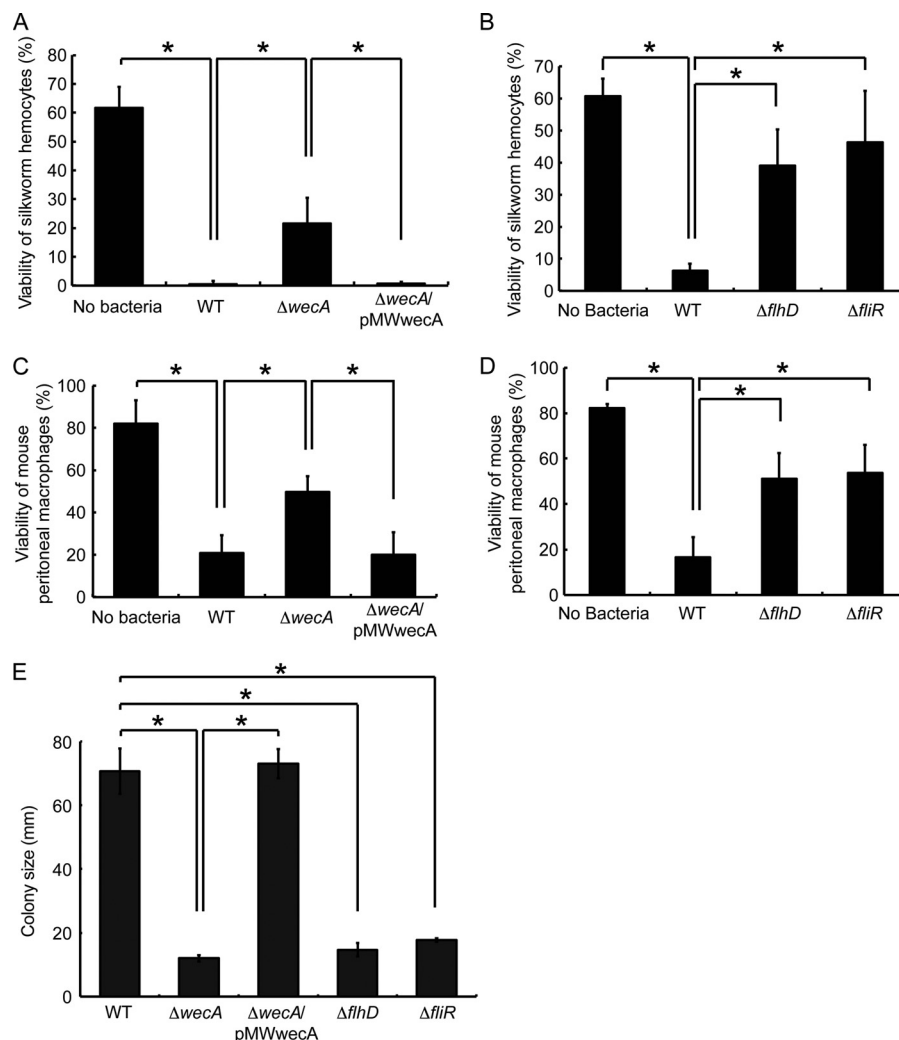


FIGURE 5. *S. marcescens* gene-disrupted mutants with impaired motility and attenuated virulence against silkworm hemocytes and mouse macrophages. A–D, immune cell killing by *S. marcescens* gene-disrupted mutants. Silkworm hemocytes (A and B) or mouse peritoneal macrophages (C and D) were suspended in PBS and incubated with either *S. marcescens* wild-type (WT), *wecA* disrupted mutant ($\Delta wecA$), *wecA* complemented strain ($\Delta wecA/pMWwecA$), *flhD* disrupted mutant ($\Delta flhD$), or *fliR* disrupted mutant ($\Delta fliR$). Data represent mean \pm S.D. of three to four experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.05$). E, motility of *S. marcescens* gene disrupted mutants on soft agar plates. Colony size of each strain was measured. Data represent mean \pm S.D. of three experiments. Statistical significance was determined by Student's *t* test (*, $p < 0.005$).

Moreover, induction of apoptosis in mouse macrophages was impaired in these gene disruptants (Fig. 5, C and D). These results suggest that LPS and flagella were involved in *S. marcescens*-induced apoptosis of silkworm hemocytes and mouse macrophages.

We then examined whether the LPS or flagella of *S. marcescens* directly induce the cell death of silkworm hemocytes. The *in vitro* viability of hemocytes was not decreased by the injection of either an LPS or flagella fraction prepared from *S. marcescens* wild-type strain within 3 h (data not shown). Moreover, LPS and flagella extracted from wild-type *S. marcescens* showed neither direct cytotoxicity nor restoration of attenuated hemocyte-killing ability in $\Delta wecA$ mutants lacking both components (supplemental Fig. S5). To further examine whether *S. marcescens* induced cell death via direct toxicity of LPS and flagella, we prepared macrophages from myeloid differentiation factor 88 (MyD88) knock-out mice that lack the potential to respond to stimulation of most Toll-like receptors. *S. marcescens* still induced cell death in macrophages obtained

from MyD88-knock-out mice (supplemental Fig. S6), suggesting that mechanisms independent of Toll-like receptor-MyD88 pathways are involved in the killing process. LPS O-antigen and flagella are required for bacterial motility (32–34). The mutants of *wecA*, *flhD*, or *fliR* constructed in our experiments were not motile on soft agar plates (Fig. 5E). These findings suggest that the motility of *S. marcescens* via LPS and flagella, and not the toxic effects of these components, is required for efficient killing of hemocytes in the host body or culture medium.

We further determined the LD₅₀ values of $\Delta wecA$, $\Delta flhD$, and $\Delta fliR$ in a silkworm infection model. Although the LD₅₀ of the parent strain (WT) was 8 ± 4 cfu/larva, the LD₅₀ of $\Delta wecA$ was $8.7 \times 10^3 \pm 5.1 \times 10^3$ cfu/larva, an approximately 10³-fold increase (Fig. 6, $p < 0.01$). In contrast, the strain complemented with a plasmid harboring the *wec* gene cluster ($\Delta wecA/pMWwecA$) had an LD₅₀ (6 ± 2 cfu/larva) similar to that of the WT (Fig. 6). The LD₅₀ of flagella mutants $\Delta flhD$ and $\Delta fliR$ was $1.6 \times 10^2 \pm 0.5 \times 10^2$ and $2.0 \times 10^2 \pm 0.8 \times 10^2$ cfu/larva, respectively, a greater than 20-fold increase (Fig. 6, $p < 0.01$).

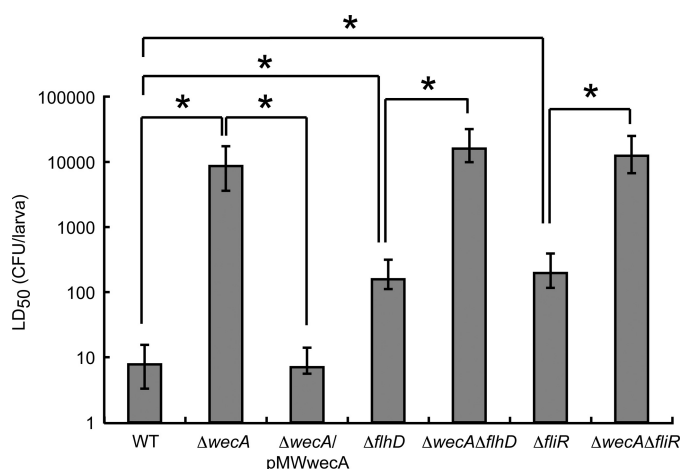


FIGURE 6. Determination of the LD₅₀ of *S. marcescens* mutants on silkworm larvae. Silkworm larvae were injected with a 2-fold serial dilution of bacterial suspension of *S. marcescens* wild-type (WT), *wecA* disrupted mutant (Δ*wecA*), *wecA* complemented strain (Δ*wecA*/pMW*wecA*), *flhD* disrupted mutant (Δ*flhD*), *fliR* disrupted mutant (Δ*fliR*), *wecA* and *flhD* double-mutant (Δ*wecA* Δ*flhD*), or *wecA* and *fliR* double-mutant (Δ*wecA* Δ*fliR*). After 16 h, the silkworm survival rate was determined and LD₅₀ values were calculated. Data represent mean ± S.D. of three to eight experiments. Statistical significance was determined by Student's *t* test (*, *p* < 0.01).

We constructed LPS- and flagella-double mutants using Δ*wecA* as the parent strain, named Δ*wecA*Δ*flhD* and Δ*wecA*Δ*fliR*, and determined the LD₅₀ values against silkworms. The LD₅₀ values of Δ*wecA*Δ*flhD* and Δ*wecA*Δ*fliR* were $1.6 \times 10^4 \pm 0.6 \times 10^4$ and $1.3 \times 10^4 \pm 0.6 \times 10^4$ cfu/larva, respectively (Fig. 6). The LD₅₀ of the double mutants was indistinguishable from that of the parent Δ*wecA*. Together with the above observation that motility was lost in the Δ*wecA*, Δ*flhD*, and Δ*fliR* single mutants (Fig. 5E), these results suggest that motility of *S. marcescens* has a critical role in silkworm killing.

DISCUSSION

Mammals protect themselves against environmental pathogens using antibody-producing systems, called acquired immunity, and other systems categorized as innate immunity. Insects like silkworms rely solely on innate immunity for self-defense. Antibody production is impaired in most aged and immunocompromised human patients, and therefore opportunistic infection models using insects as host animals are considered to mimic the pathology of compromised humans. Here, we analyzed the virulence mechanism of a human opportunistic pathogen, *S. marcescens*, in a silkworm infection model. The data obtained suggest that the high virulence of *S. marcescens* against silkworm larvae is due to the death of host immune cells, thereby suppressing systemic immune responses.

Cell death pathways in higher organisms are required not only for morphologic formation during developmental stages, but also for immunologic reactions (35). Host cells that are damaged by invading pathogens actively undergo cell death to avoid the systemic spreading of infection (35). Conversely, at the same time, the loss of immune cells seems to slow down the elimination of pathogens in tissues and bloodstream, leading to serious problems such as excessive pathogen proliferation. *Aeromonas hydrophila* (36) and Group A *Streptococcus* (37), causative agents of diarrhea and sepsis, induce immune cell

death, inhibit cytokine production, and promote bacterial growth in mammalian tissues. Although *S. marcescens* causes apoptosis-like cell death in cultured Chinese hamster ovary cells (38), there are no reports suggesting that *S. marcescens* escapes host immunity via the killing of immune cells. The present study is the first to demonstrate that *S. marcescens* has a strategy to disrupt the immune system to effectively kill the host.

There are few reports that insect cytokines possess immunomodulating function similar to mammalian cytokines. We previously demonstrated that PP, the multifunctional peptide in the hemolymph of silkworms, contributes to the activation of various immune responses and host protection against infections (17, 18). In addition, we reported that hemocytes stimulated by microbial infections are necessary for activating the serine proteases responsible for proteolytic cleavage of the inactive PP precursor to generate the active form of PP (17). The present results suggest that, in the process of *S. marcescens* infection, hemocytes required for PP activation are killed and therefore the onset of acute immune responses including AMP production and phagocytosis of bacteria are suppressed, leading to severe impairment of host resistance. In mammals, many types of immune cells, such as macrophages and natural killer cells, have critical roles in the early stages of infection by producing various cytokines such as tumor necrosis factor-α and interleukins. In our experiments, we found that *S. marcescens* induces JNK- and caspase-dependent apoptotic cell death in mouse peritoneal macrophages *in vitro*. Thus, we speculated that *S. marcescens* exerts virulence by a common mechanism in insects and mammals; killing host immune cells to impair cytokine production and other immune responses. Although it seems that most aspects of innate immunity are conserved among species, there are some differences between invertebrate and vertebrate immune systems. Therefore, carefully controlled experiments using alternative animal models should be performed to elucidate the pathogenesis of *S. marcescens*.

Factors involved in cell death reactions are highly conserved among species (27, 28). JNK (39) and caspases (40) have been identified in silkworms as well as in other organisms. Moreover, stress response pathways such as p38 and PI3K are also conserved and are involved in both immune responses (41) and apoptosis (42). Our experiments using pharmacologic inhibitors suggested that activation of JNK but not p38 or PI3K was required for host cell killing by *S. marcescens*; the possible involvement of other stress-signaling pathways, however, is not ruled out. Some pathogens exert virulence by overactivating stress factors in the host cells. For example, *Francisella tularensis* causes excess caspase-3 activation in mouse organs (43). *Pseudomonas aeruginosa* activates the JNK-dependent caspase pathway through a type III secretion system (44, 45). We recently reported that live and heat-killed *Porphyromonas gingivalis*, a human orthodontic pathogen, causes excessive activation of an immune reaction called melanization in the silkworm hemolymph, leading to the overproduction of reactive oxygen species and activation of caspases in larval tissues (46). In contrast, we did not observe extensive melanization in the hemolymph of silkworms injected with live *S. marcescens*. Thus, the underlying mechanism of apoptosis induction seems to differ

between *P. gingivalis* and *S. marcescens*. Exotoxins (47) and cell wall components (48) of certain bacteria induce host cell death. In the present experiments, acute killing of hemocytes by the culture supernatant and heat-killed cells of *S. marcescens* was not observed. Therefore, we considered a novel mechanism in which *S. marcescens* induces hemocyte death by direct interaction. Although the type III secretion system is one such mechanism, we know of no reports to date that have examined its presence and function in *Serratia*. On the other hand, *Serratia* ShlB protein is known as a transporter that mediates two-partner secretion (49). Identification of the responsible factors on the surface of the bacteria is currently underway in our laboratory.

Kurz and colleagues (24) performed *in vivo* screening using *Caenorhabditis elegans* to search for *S. marcescens* virulence factors and found that gene mutations of enzymes involved in LPS production attenuated the virulence of *S. marcescens* in nematodes. Whether these factors contribute to cell death in immune cells, however, has yet to be determined. In this report, we screened for mutants of *S. marcescens* with decreased killing abilities against hemocytes and identified virulence genes required for the apoptosis induction of hemocytes. Among those, *bsmB* is required for adhesion to solid surfaces and biofilm formation in a closely related bacterium *Serratia liquifaciens* (50, 51), whereas its role in virulence against host cells and animals is not yet clear. Another gene, *citC*, encoding citrate lyase ligase, which is an enzyme involved in anaerobic citrate metabolism in some bacteria (52), might contribute to intracellular infection of *Shigella flexneri* to HeLa cells (53). We identified several other genes whose functions have not been fully characterized. Studies of the functions of those genes might help to clarify the cell death-inducing mechanism of *S. marcescens* and other microbes with homologous genes.

Mutants of the *wecA* gene (responsible for LPS synthesis) and the *flhD* and *fliR* genes (responsible for flagella synthesis) had severely impaired host cell-killing phenotypes. These genes contribute to motility and virulence processes in several different bacterial species (32, 54). Therefore, we concluded that *S. marcescens* motility, which is dependent on LPS and flagella, was critical to the high pathogenicity of this bacterium on silkworms. Other possibilities, however, could not be ruled out. LPS and flagella are both major bulky complexes on the bacterial surface that could affect extracellular secretion and adhesion (55, 56). Therefore, the mutants we obtained might have impaired secretion and adhesion, which could attenuate killing ability. In our experimental conditions, neither the LPS nor flagella fraction prepared from *S. marcescens* caused cell death, but it is still possible that the intact forms of these components on the bacterial surface possess cytotoxic activity and that this activity was lost during sample preparation. Moreover, LPS might show cytotoxic effects via mechanisms other than direct stimulation of Toll-like receptor-MyD88 pathways, because complex activation mechanisms of LPS-dependent apoptosis involving other receptors, such as scavenger receptors, have been reported in mouse macrophages (57). Further studies are required to identify the executive factor and downstream mechanisms of host cell killing.

The LD₅₀ of the *wecA* gene disrupted mutant (Δ *wecA*) was more than 10-fold higher than that of the *flhD* or *fliR* mutants (Δ *flhD* or Δ *fliR*), whereas those mutants were completely immotile on soft agar plates (Fig. 5E). Although we concluded that the motility of *S. marcescens* is important for silkworm killing, these results suggest that *wecA* might be involved in processes other than motility. In contrast to the significantly different killing effects of these mutants on silkworms, the numbers of mutant bacteria required for hemocyte killing were comparable (supplemental Fig. S7). Thus, the above difference in LD₅₀ against silkworms does not seem to be explained by differences in the hemocyte killing ability, but could be due to other factors ("host killing factors"), likely regulated by *wecA*-dependent O-glycosylation, that directly cause silkworm death. Injection of a higher concentration of *S. marcescens* culture supernatant than that shown in Fig. 1 killed silkworms (whereas a higher concentration of supernatant still did not induce hemocyte cell death, similar to that shown in Fig. 2A), and that its host killing activity was partially dependent on *wecA*.³ Identification of the *wecA*-dependent host-killing factor in the supernatant is now underway. Determining the functional difference between *wecA* and flagella synthetic genes (*flhD* and *fliR*) in regard to animal killing might lead to better understanding of the virulence mechanism of *S. marcescens*.

Acknowledgments—We are grateful to Dr. Shizuo Akira (Osaka University), and Dr. Kaori Denda-Nagai, Dr. Nobuaki Higashi, and Dr. Tatsuo Irimura (University of Tokyo) for providing the MyD88 knock-out mice.

REFERENCES

1. Hejazi, A., and Falkner, F. R. (1997) *Serratia marcescens*. *J. Med. Microbiol.* **46**, 903–912
2. Villari, P., Crispino, M., Salvadori, A., and Scarcella, A. (2001) Molecular epidemiology of an outbreak of *Serratia marcescens* in a neonatal intensive care unit. *Infect. Control. Hosp. Epidemiol.* **22**, 630–634
3. Maragakis, L. L., Winkler, A., Tucker, M. G., Cosgrove, S. E., Ross, T., Lawson, E., Carroll, K. C., and Perl, T. M. (2008) Outbreak of multidrug-resistant *Serratia marcescens* infection in a neonatal intensive care unit. *Infect. Control Hosp. Epidemiol.* **29**, 418–423
4. Sifri, C. D., Begun, J., and Ausubel, F. M. (2005) The worm has turned. Microbial virulence modeled in *Caenorhabditis elegans*. *Trends Microbiol.* **13**, 119–127
5. Scully, L. R., and Bidochka, M. J. (2006) Developing insect models for the study of current and emerging human pathogens. *FEMS Microbiol. Lett.* **263**, 1–9
6. Kaito, C., Akimitsu, N., Watanabe, H., and Sekimizu, K. (2002) Silkworm larvae as an animal model of bacterial infection pathogenic to humans. *Microb. Pathog.* **32**, 183–190
7. Hamamoto, H., Kurokawa, K., Kaito, C., Kamura, K., Manitra Razanajato, I., Kusuhara, H., Santa, T., and Sekimizu, K. (2004) Quantitative evaluation of the therapeutic effects of antibiotics using silkworms infected with human pathogenic microorganisms. *Antimicrob. Agents Chemother.* **48**, 774–779
8. Hamamoto, H., and Sekimizu, K. (2005) Evaluation of the therapeutic effects of antibiotics using silkworm as an animal model. *Res. Adv. Antimicrob. Agents Chemother.* **5**, 1–23
9. Kaito, C., Kurokawa, K., Matsumoto, Y., Terao, Y., Kawabata, S., Hamada, S., and Sekimizu, K. (2005) Silkworm pathogenic bacteria infection model

³ K. Ishii, H. Hamamoto, and K. Sekimizu, unpublished data.

- for identification of novel virulence genes. *Mol. Microbiol.* **56**, 934–944
10. Kaito, C., and Sekimizu, K. (2007) A silkworm model of pathogenic bacterial infection. *Drug Discov. Ther.* **1**, 89–93
11. Usui, K., Miyazaki, S., Kaito, C., and Sekimizu, K. (2009) Purification of a soil bacteria exotoxin using silkworm toxicity to measure specific activity. *Microb. Pathog.* **46**, 59–62
12. Kaito, C., Usui, K., Kyuma, T., and Sekimizu, K. (2011) Isolation of mammalian pathogenic bacteria using silkworms. *Drug Discov. Ther.* **5**, 66–70
13. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318
14. Tanaka, H., Ishibashi, J., Fujita, K., Nakajima, Y., Sagisaka, A., Tomimoto, K., Suzuki, N., Yoshiyama, M., Kaneko, Y., Iwasaki, T., Sunagawa, T., Yamaji, K., Asaoka, A., Mita, K., and Yamakawa, M. (2008) A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. *Insect Biochem. Mol. Biol.* **38**, 1087–1110
15. Ha Lee, J., Hee Lee, I., Noda, H., Mita, K., and Taniai, K. (2007) Verification of elicitor efficacy of lipopolysaccharides and peptidoglycans on antibacterial peptide gene expression in *Bombyx mori*. *Insect Biochem. Mol. Biol.* **37**, 1338–1347
16. Taniai, K., Wago, H., and Yamakawa, M. (1997) In vitro phagocytosis of *Escherichia coli* and release of lipopolysaccharide by adhering hemocytes of the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **231**, 623–627
17. Ishii, K., Hamamoto, H., Kamimura, M., and Sekimizu, K. (2008) Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. *J. Biol. Chem.* **283**, 2185–2191
18. Ishii, K., Hamamoto, H., Kamimura, M., Nakamura, Y., Noda, H., Imamura, K., Mita, K., and Sekimizu, K. (2010) Insect cytokine paralytic peptide (PP) induces cellular and humoral immune responses in the silkworm *Bombyx mori*. *J. Biol. Chem.* **285**, 28635–28642
19. Nakahara, Y., Kanamori, Y., Kiuchi, M., and Kamimura, M. (2003) Effects of silkworm paralytic peptide on *in vitro* hematopoiesis and plasmacytocyte spreading. *Arch. Insect. Biochem. Physiol.* **52**, 163–174
20. Sekimizu, K., Larranaga, J., Hamamoto, H., Sekine, M., Furuchi, T., Katane, M., Homma, H., and Matsuki, N. (2005) D-Glutamic acid-induced muscle contraction in the silkworm, *Bombyx mori*. *J. Biochem.* **137**, 199–203
21. Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyogi, M., Ferrer, S., and Regue, M. (1997) Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J. Bacteriol.* **179**, 7111–7117
22. de Lorenzo, V., Herrero, M., Jakubzik, U., and Timmis, K. N. (1990) Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in Gram-negative eubacteria. *J. Bacteriol.* **172**, 6568–6572
23. Toratani, T., Shoji, T., Ikehara, T., Suzuki, K., and Watanabe, T. (2008) The importance of chitinase and N-acetylglucosamine (GlcNAc) uptake in *N,N'*-diacetylchitobiose ((GlcNAc)₂) utilization by *Serratia marcescens* 2170. *Microbiology* **154**, 1326–1332
24. Kurz, C. L., Chauvet, S., Andr s, E., Aurouze, M., Vallet, I., Michel, G. P., Uh, M., Celli, J., Filloux, A., De Bentzmann, S., Steinmetz, I., Hoffmann, J. A., Finlay, B. B., Gorvel, J. P., Ferrandon, D., and Ewbank, J. J. (2003) Virulence factors of the human opportunistic pathogen *Serratia marcescens* identified by *in vivo* screening. *EMBO J.* **22**, 1451–1460
25. Nishida, S., Mizushima, T., Miki, T., and Sekimizu, K. (1997) Immobile phenotype of an *Escherichia coli* mutant lacking the histone-like protein HU. *FEMS Microbiol. Lett.* **150**, 297–301
26. Nehme, N. T., Li geois, S., Kele, B., Giammarinaro, P., Pradel, E., Hoffmann, J. A., Ewbank, J. J., and Ferrandon, D. (2007) A model of bacterial intestinal infections in *Drosophila melanogaster*. *PLoS Pathog.* **3**, e173
27. Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X., and Vandenabeele, P. (2002) Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ.* **9**, 358–361
28. Kanuka, H., Hisahara, S., Sawamoto, K., Shoji, S., Okano, H., and Miura, M. (1999) Proapoptotic activity of *Caenorhabditis elegans* CED-4 protein in *Drosophila*. Implicated mechanisms for caspase activation. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 145–150
29. Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T., and Miura, M. (2002) Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. *EMBO J.* **21**, 3009–3018
30. Moreno, E., Yan, M., and Basler, K. (2002) Evolution of TNF signaling mechanisms. JNK-dependent apoptosis triggered by Eiger, the *Drosophila* homolog of the TNF superfamily. *Curr. Biol.* **12**, 1263–1268
31. Morgenstein, R. M., Clemmer, K. M., and Rather, P. N. (2010) Loss of the waaL O-antigen ligase prevents surface activation of the flagellar gene cascade in *Proteus mirabilis*. *J. Bacteriol.* **192**, 3213–3221
32. Easom, C. A., and Clarke, D. J. (2008) Motility is required for the competitive fitness of entomopathogenic *Photobacterium luminescens* during insect infection. *BMC Microbiol.* **8**, 168
33. Tabei, S. M., Hitchen, P. G., Day-Williams, M. J., Merino, S., Vart, R., Pang, P. C., Horsburgh, G. J., Viches, S., Wilhelms, M., Tom s, J. M., Dell, A., and Shaw, J. G. (2009) An *Aeromonas caviae* genomic island is required for both O-antigen lipopolysaccharide biosynthesis and flagellin glycosylation. *J. Bacteriol.* **191**, 2851–2863
34. Abeyratne, P. D., Daniels, C., Poon, K. K., Matewish, M. J., and Lam, J. S. (2005) Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J. Bacteriol.* **187**, 3002–3012
35. Labb , K., and Saleh, M. (2008) Cell death in the host response to infection. *Cell Death Differ.* **15**, 1339–1349
36. Majumdar, T., Chattopadhyay, P., Saha, D. R., Sau, S., and Mazumder, S. (2009) Virulence plasmid of *Aeromonas hydrophila* induces macrophage apoptosis and helps in developing systemic infection in mice. *Microb. Pathog.* **46**, 98–107
37. Timmer, A. M., Timmer, J. C., Pence, M. A., Hsu, L. C., Ghochani, M., Frey, T. G., Karin, M., Salvesen, G. S., and Nizet, V. (2009) Streptolysin O promotes group A *Streptococcus* immune evasion by accelerated macrophage apoptosis. *J. Biol. Chem.* **284**, 862–871
38. Carbonell, G. V., Falc n, R., Yamada, A. T., da Fonseca, B. A., and Yano, T. (2004) Morphological and intracellular alterations induced by *Serratia marcescens* cytotoxin. *Res. Microbiol.* **155**, 25–30
39. Katsuma, S., Mita, K., and Shimada, T. (2007) ERK- and JNK-dependent signaling pathways contribute to *Bombyx mori* nucleopolyhedrovirus infection. *J. Virol.* **81**, 13700–13709
40. Pei, Z., Reske, G., Huang, Q., Hammock, B. D., Qi, Y., and Chejanovsky, N. (2002) Characterization of the apoptosis suppressor protein P49 from the *Spodoptera littoralis* nucleopolyhedrovirus. *J. Biol. Chem.* **277**, 48677–48684
41. Shinzawa, N., Nelson, B., Aonuma, H., Okado, K., Fukumoto, S., Miura, M., and Kanuka, H. (2009) p38 MAPK-dependent phagocytic encapsulation confers infection tolerance in *Drosophila*. *Cell Host Microbe* **6**, 244–252
42. Cho, K. S., Lee, J. H., Kim, S., Kim, D., Koh, H., Lee, J., Kim, C., Kim, J., and Chung, J. (2001) *Drosophila* phosphoinositide-dependent kinase-1 regulates apoptosis and growth via the phosphoinositide 3-kinase-dependent signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 6144–6149
43. Wickstr m, J. R., Bokhari, S. M., Fischer, J. L., Pinson, D. M., Yeh, H. W., Horvat, R. T., and Parmely, M. J. (2009) *Francisella tularensis* induces extensive caspase-3 activation and apoptotic cell death in the tissues of infected mice. *Infect. Immun.* **77**, 4827–4836
44. Miao, E. A., Ernst, R. K., Dors, M., Mao, D. P., and Aderem, A. (2008) *Pseudomonas aeruginosa* activates caspase 1 through Ipaf. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 2562–2567
45. Jia, J., Alaoui-El-Azher, M., Chow, M., Chambers, T. C., Baker, H., and Jin, S. (2003) c-Jun NH₂-terminal kinase-mediated signaling is essential for *Pseudomonas aeruginosa* ExoS-induced apoptosis. *Infect. Immun.* **71**, 3361–3370
46. Ishii, K., Hamamoto, H., Imamura, K., Adachi, T., Shoji, M., Nakayama, K., and Sekimizu, K. (2010) *Porphyromonas gingivalis* peptidoglycans induce excessive activation of the innate immune system in silkworm larvae. *J. Biol. Chem.* **285**, 33338–33347
47. Liu, G. Y., Doran, K. S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L., and Nizet, V. (2004) Sword and shield. Linked group B streptococcal β -hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14491–14496
48. Dao, D. N., Kremer, L., Gu rardel, Y., Molano, A., Jacobs, W. R., Jr., Porcelli, S. A., and Briken, V. (2004) *Mycobacterium tuberculosis* lipomannan

***S. marcescens* Suppresses Innate Immunity**

- induces apoptosis and interleukin-12 production in macrophages. *Infect. Immun.* **72**, 2067–2074
49. Kostakioti, M., Newman, C. L., Thanassi, D. G., and Stathopoulos, C. (2005) Mechanisms of protein export across the bacterial outer membrane. *J. Bacteriol.* **187**, 4306–4314
50. Labbate, M., Queck, S. Y., Koh, K. S., Rice, S. A., Givskov, M., and Kjelleberg, S. (2004) Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. *J. Bacteriol.* **186**, 692–698
51. Labbate, M., Zhu, H., Thung, L., Bandara, R., Larsen, M. R., Willcox, M. D., Givskov, M., Rice, S. A., and Kjelleberg, S. (2007) Quorum-sensing regulation of adhesion in *Serratia marcescens* MG1 is surface dependent. *J. Bacteriol.* **189**, 2702–2711
52. Bott, M., and Dimroth, P. (1994) *Klebsiella pneumoniae* genes for citrate lyase and citrate lyase ligase. Localization, sequencing, and expression. *Mol. Microbiol.* **14**, 347–356
53. Mita, K., Kasahara, M., Sasaki, S., Nagayasu, Y., Yamada, T., Kanamori, H., Namiki, N., Kitagawa, M., Yamashita, H., Yasukochi, Y., Kadono-Okuda, K., Yamamoto, K., Ajimura, M., Ravikumar, G., Shimomura, M., Nagamura, Y., Shin-I, T., Abe, H., Shimada, T., Morishita, S., and Sasaki, T. (2004) The genome sequence of silkworm, *Bombyx mori*. *DNA Res.* **11**, 27–35
54. Forbes, S. J., Eschmann, M., and Mantis, N. J. (2008) Inhibition of *Salmonella enterica* serovar typhimurium motility and entry into epithelial cells by a protective antilipopolysaccharide monoclonal immunoglobulin A antibody. *Infect. Immun.* **76**, 4137–4144
55. DeFlaun, M. F., Oppenheimer, S. R., Streger, S., Condee, C. W., and Fletcher, M. (1999) Alterations in adhesion, transport, and membrane characteristics in an adhesion-deficient pseudomonad. *Appl. Environ. Microbiol.* **65**, 759–765
56. Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L., and Kolter, R. (2001) The absence of a flagellum leads to altered colony morphology, biofilm development, and virulence in *Vibrio cholerae* O139. *Mol. Microbiol.* **39**, 223–235
57. Seimon, T. A., Obstfeld, A., Moore, K. J., Golenbock, D. T., and Tabas, I. (2006) Combinatorial pattern recognition receptor signaling alters the balance of life and death in macrophages. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 19794–19799